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EXHIBIT A

REVIEW

Factors affecting xylanase functionality in the degradation of arabinoxylans

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Abstract *Endo*-β-1,4-xylanases are key enzymes in the degradation of arabinoxylans, the main non-starch polysaccharides from grain cell walls. Due to the heterogeneity of arabinoxylans, xylanases with different characteristics are required in industrial applications but the choice of the enzyme is still largely empirical. Although the classification into glycoside hydrolase families greatly helped to derive mechanistic information on the catalytic and substrate specificity of xylanases, other factors e.g. their sensitivity to endogenous inhibitors, the presence of carbohydrate-binding module(s) and their degree of selectivity towards soluble versus insoluble substrate may play a role in determining the functionality of these enzymes in the degradation of arabinoxylans.

Keywords Arabinoxylan · Glycoside hydrolases · Substrate specificity · Xylanase inhibitors · Xylan binding domains

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Introduction

Endo-(1,4)-β-xylanases (xylanases; EC 3.2.1.8) depolymerize the xylan backbone, a significant component of the plant cell wall, by cleaving the β-(1,4) glycosidic bonds between D-xylose residues in the main chain. The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases that differ in their physico-chemical properties, structure, mode of action and substrate specificities. A classification system for glycoside hydrolases (GH) has been established based on amino acid sequence similarities (Coutinho and Henrissat 1999) and, at present, at least 112 different families have been identified (http://afmb.cnrs-mrs.fr/CAZY/). The majority of xylanases cluster into families 10 and 11 but xylanases have also been classified in GH families 5, 7, 8 and 43 (for a review, see Collins et al. 2005).

In the plant cell wall, xylan can be substituted by different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl, and glucuronic acid residues. Arabinoxylans (AXs) are the main non-starch polysaccharides in wheat and other cereals (for a review on cell wall polysaccharides, see Waldron and Faulds 2007). These cell wall components amount to 2–4% of wheat flour and are involved to a great extent in second transformation processes. The extent and the nature of xylan decorations in AX vary between different species, the xylan backbone is either unsubstituted or mono- or disubstituted with single α-L-arabinofuranose residues at the *O*-2 and/or

O-3 position(s) and some of these residues are ester-linked with a ferulic acid moiety at position *O*-5 (Saulnier et al. 2007). Xylanases drastically change the structure and physicochemical properties of the AX population. On one hand they solubilise water-unextractable AX (WU-AX) which leads to loss in water-holding capacity and, on the other hand, they degrade water-extractable AX (WE-AX) and solubilised AX (S-AX), which leads to a decrease in viscosity (Saulnier et al. 2007; Waldron and Faulds 2007). Therefore, cereal based food and feed sectors are directly concerned by the use of xylanases with targeted properties. Different xylanases are routinely used in bread making (Courtin et al. 1999), pasta processing (Ingelbrecht et al. 2001), wheat gluten–starch separation (Christophersen et al. 1997) and as supplements in animal feed production (Choct et al. 2004). In these applications, the observed effects are largely dependent on the variability in xylanase specificity towards AXs, which can be attributed to different factors such as their GH family origin, sensitivity to protein inhibitors, modular architecture, and substrate selectivity.

GH family origin

The Carbohydrate-Active enZymes database (CAZy) provides a continuously updated list of the glycoside hydrolase families (http://afmb.cnrs-mrs.fr/CAZY/). Because there is a direct relationship between sequence and folding similarities, such a classification (i) reflects the structural features of these enzymes better than their sole substrate specificity, (ii) helps to reveal the evolutionary relationships between these enzymes, and (iii) provides a convenient tool to derive mechanistic information.

To date, more than 39 three-dimensional (3D) structures of xylanases have been solved, mainly from the two main xylanase families, GH10 and GH11 (Fig. 1). All GH11 structures present a "jelly-roll" fold in which two large β-pleated sheets and one α-helix form a structure resembling that of a partially closed right hand, with the active site "palm" formed by the cleft between the "fingers" and the "thumb" (Törrönen and Rouvinen 1997). The $(\beta/\alpha)_8$ barrel structure of GH10 members has been likened to a "salad bowl", with one face of the molecule having a large radius due to an elaborate loop architecture, whereas the opposite face consists of simple α/β turns (Harris et al. 1994). This is similar to the fold described for GH5 enzymes, e.g. xylanase (XYNA) from *Erwinia chrysanthemi* (1NOF.pdb) (Larson et al. 2003). The structure of a psychrophilic GH8 xylanase from *Pseudoalteromonas haloplanktis* (1H12.pdb) folds as a $(\alpha/\alpha)_6$ barrel formed by six inner and six outer helices (Van Petegem et al. 2003). No 3D structure of GH7 and GH43 xylanases is available but members of these GH families fold as a β-jelly roll and a β-propeller fold, respectively.

Families 5, 7, 10 and 11 contain enzymes which catalyse hydrolysis with retention of anomeric configuration with two glutamate residues located at opposite sides of the sugar plane being implicated in the catalytic mechanism (Coutinho and Henrissat 1999). This involves a double-displacement mechanism. In contrast, enzymes in families 8 and 43 typically operate via inversion of the anomeric centre with a glutamate and aspartate that are believed to be the catalytic residues. They function by single-displacement reaction (Nurizzo et al. 2002).

Although GH10 and GH11 have been studied in detail, the catalytic properties of recently discovered xylanases from GH families 5, 7, 8, and 43 remains very limited. The GH7 enzyme from *Trichoderma reesei* is considered as a non-specific *endo*-β-1,4 glucanase (Kleywegt et al. 1997) and the GH43 enzyme from *Paenibacillus polymyxa* display both xylanase and α-L-arabinofuranosidase activities (Gosalbes et al. 1991). The GH5 enzyme from *E. chrysanthemi* is specialized for hydrolysis of 4-*O*-methyl-D-glucuronoxylan or its acetylated counterparts and does not attack other types of xylans, linear β-1,4-*xylo*-oligosaccharides or esterified aldouronic acids (Vršanská et al. 2007). The cold-adapted GH8 enzyme is a "true xylanase" but with a marked preference for linear mixed linkage (β-1,3 and β-1,4) xylan (Collins et al. 2002). The GH11 family is monospecific, it consists only of "true xylanases" displaying exclusive substrate specificity towards D-xylose containing substrates. These enzymes have a lower catalytic versatility than GH10 xylanases and the products of their action can be further hydrolysed by GH10 enzymes (Collins et al. 2005). For GH11 xylanases, the main end products from the hydrolysis of *xylo*-oligosaccharides are xylobiose and xylotriose (e.g. Berrin et al. 2007) whereas GH10 xylanases are able to release xylose as end product (e.g. Charnock et al. 1997). One interesting feature of GH10 xylanase is that,

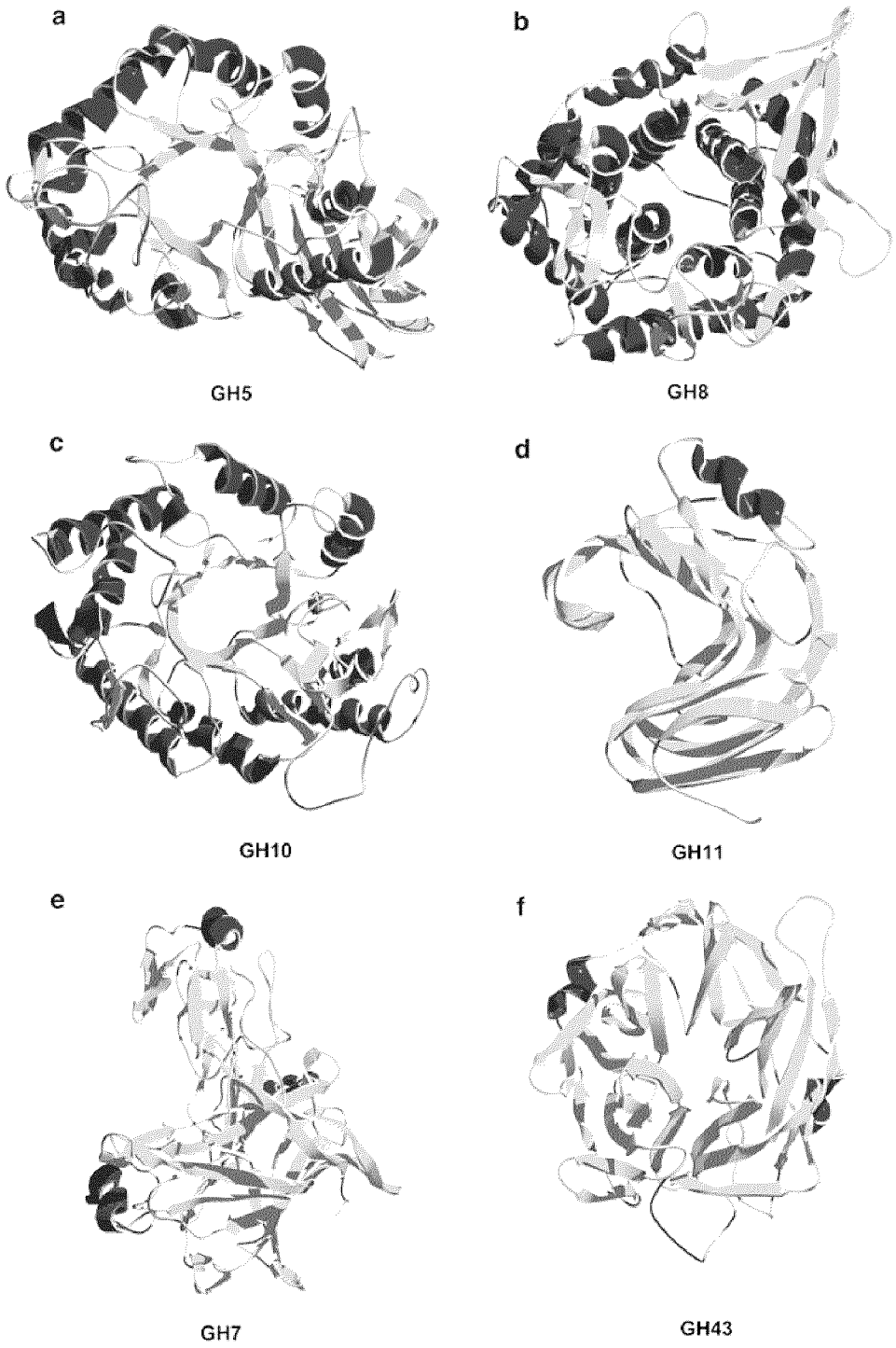

Fig. 1 Structures of xylanases from glycoside hydrolase families. (**a**) *Erwinia chrysanthemi* family 5 xylanase (1NOF.pdb; Larson et al. 2003); (**b**) *Pseudoalteromonas haloplanktis* family 8 xylanase (1H12.pdb; Van Petegem et al. 2003); (**c**) *Pseudomonas fluorescens* family 10 xylanase (1CLX.pdb; Harris et al. 1994); (**d**) *Penicillium funiculosum* family 11 xylanase (1TE1.pdb; Payan et al. 2004); (**e**) *Trichoderma reesei* family 7 endoglucanase (1GE1.pdb; Kleywegt et al. 1997); (**f**) *Cellvibrio japonicus* family 43 arabinanase (1GYD.pdb; Nurizzo et al. 2002). This figure was prepared using Swiss PDB-Viewer (http://expasy.ch/spdbv/)

in addition to their xylanolytic activity, they display a range of tolerance for glucose-derived substrates, such as aryl cellobiosides (Collins et al. 2005). In further contrast to GH10 xylanases, but in common with GH8 cold-adapted xylanase, GH11 enzymes are most active on long chain *xylo*-oligosaccharides. The substrate specificity of xylanases is reflected by the structural features of their active site. Each xylose is accommodated in a subsite, prefixed by (−) and (+), depending on whether it binds the glycone or aglycone regions of the substrate, respectively. The cleavage by definition takes place between subsites (−1) and (+1) (Biely et al. 1981; Davies et al. 1997). The size of the substrate binding cleft defines the degree of polymerisation (DP) of

xylo-oligosaccharide end products. Kinetic and structural investigations of GH11 xylanases indicate that their active sites potentially have up to three (−) subsites and three (+) subsites (Janis et al. 2005). In contrast, GH7 and GH10 xylanases have four to five susbites (Biely et al. 1997; Collins et al. 2005), although, the topology of the substrate binding clefts of GH10 enzymes is very variable (Charnock et al. 1998). The cold-adapted GH8 xylanase has a large substrate binding cleft containing at least six xylose binding residues, with the catalytic site in the middle (Collins et al. 2002).

Among these GH families, only GH10 and GH11 xylanases hydrolyse AXs (Kormelink et al. 1993a, b). It is generally accepted that GH11 xylanases preferentially cleave in unsubstituted regions of the AX backbone, whereas GH10 cleave in decorated regions, being less hampered by the presence of 4-*O*-methyl-D-glucuronate, acetate and α-L-arabinofuranosyl substituents along the xylan backbone (Biely et al. 1997). This is also reflected by the shape of their active site. The GH10 xylanase active site is a shallow groove which mirrors a specificity towards a lower number of unsubstituted consecutive xylose units whereas GH11 xylanases show higher affinity towards a larger number of unsubstituted consecutive xylose units because of their cleft-shaped active site. A feature that distinguishes GH10 and GH11 xylanases is the nature of the reaction products released from decorated xylans. GH11 xylanases produce oligosaccharides containing unsubstituted *xylo*-oligosaccharides both at the aglycone (+1) and glycone (−1) subsites, although some decorated *xylo*-oligosaccharides were identified as limit products (Maslen et al. 2007). These structures are expected based on previous studies on xylanases from GH11, which are only able to hydrolyze unsubstituted regions of xylan (Kormelink et al. 1993a, b; Biely et al. 1997). Recent crystallographic studies have provided insight into the mechanism by which the GH10 and GH11 enzymes are able to hydrolyse decorated xylans (Pell et al. 2004; Vardakou et al. 2005, 2008) but whether xylan decorations contribute to substrate recognition or are simply accommodated by xylanases appears to depend on the nature of the xylanase. *Ta*Xyn10 xylanase from *Thermoascus aurantiacus* displayed four-fold more activity against xylotriose in which the non-reducing moiety is linked to an arabinose side-chain, compared to the undecorated form of the oligosaccharide (Vardakou et al. 2005). However more kinetic studies are necessary to confirm the ability of GH10 and GH11 xylanases to recognize arabinofuranosyl residues.

Substrate selectivity

The effect of exogenous xylanases used as process aids in cereal-based products depends on the mode of action of the xylanase, and is influenced by variations in the primary structure of AX, e.g. the degree of substitution, and by the availability of AX as WE-AX or WU-AX forms. While WU-AX are retained in the cell wall by covalent and noncovalent interactions with other AX molecules and cell wall constituents such as protein, lignin, cellulose or β-glucan, WE-AX are thought to be loosely bound at the surface of these cell walls. In wheat endosperm, about 25% of the AXs are WE-AX with an average arabinose to xylose ratio (A/X) of 0.6 although large natural variations in the content and the structural features of WE-AX occur among wheat cultivars (Delcour et al. 1999). The structural features of WE-AX are well documented, but less information is available for WU-AX, which represent the major part of AX in cell walls of the endosperm. WU-AXs have been studied after alkaline extraction (AE-AX) (Gruppen et al. 1992a, b, 1993a, b; for a review see Saulnier et al. 2007) and xylanase extraction (XE-AX) (Ordaz-Ortiz and Saulnier 2005). The structure of WU-AX is very close to that of WE-AX but the average molecular weight and A/X ratio are slightly higher for WU-AX than for WE-AX (Izydorczyk and Biliaderis 1995).

The relative activity of xylanases towards WU-AX and WE-AX substrates, referred to as xylanase substrate selectivity (Moers et al. 2005), impacts on the enzyme functionality in cereal-based biotechnological processes. For instance, a GH11 *B. subtilis* xylanase, which preferentially hydrolyses WU-AX and leaves WE-AX and solubilised AX (S-AX) unharmed, beneficially affects bread loaf volume (Courtin et al. 2001; Courtin and Delcour 2001). The molecular weight of WE-AX is thus a key factor in the impact of xylanases in bread-making. Only a limited number of xylanases that are able to solubilise high molecular weight AX from insoluble AX with very little degradation of WE-AX are used in bread making. On the other hand, the use of xylanases with different substrate selectivities (GH10 *A. aculeatus*

Table 1 CBMs of known structure linked to xylanases

Family	Type	Fold	Protein	PDB code
CBM-2	B	β-Sandwich	Xylanase 10A (*Cellulomonas fini*)	1EXG
	B	β-Sandwich	Xylanase 11A (*Cellulomonas fini*)	2XBD
	B	β-Sandwich	Xylanase 11A (*Cellulomonas fini*)	1HEH
CBM-4	B	β-Sandwich	Xylanase 10A (*Rhodothermus marinus*)	1K45
CBM-6	B	β-Sandwich	Xylanase 11A (*Clostridium thermocellum*)	1UXX
	B	β-Sandwich	Xylanase 11A (*Clostridium stercorarium*)	1NAE
	B	β-Sandwich	Xylanase 11A (*Clostridium stercorarium*)	1UY4
CBM-9	C	β-Sandwich	Xylanase 10A (*Thermotoga maritima*)	1I8A
CBM-10	A	OB fold[a]	Xylanase 10A (*Cellvibrio japonicus*)	1QLD
CBM-13	C	β-Trefoil	Xylanase 10A (*Streptomyces olivaceovidris*)	1XYF
	C	β-Trefoil	Xylanase 10A (*Streptomyces lividans*)	1MC9
CBM-15	B	β-Sandwich	Xylanase 10C (*Cellvibrio japonicus*)	1GNY
CBM-22	B	β-Sandwich	Xylanase 10B (*Clostridium thermocellum*)	1DYO
CBM-36	B	β-Sandwich	Xylanase 43A (*Paenibacillus polymyxa*)	1UX7
	B	β-Sandwich	Xylanase 11J (*Bacillus sp.*)	2DCJ

[a] OB, oligonucleotide/oligosaccharide binding

vs. GH11 *B. subtilis*) showed that the degradation of WE-AX and S-AX to low molecular weight molecules rather than the conversion of WU-AX to high molecular weight WE-AX was the main factor influencing dough syruping (Courtin et al. 1999). The GH10 xylanase of *A. aculeatus*, which preferentially degrades WE-AX and S-AX, is also used to improve gluten agglomeration in industrial wheat gluten separation (Christophersen et al. 1997; Frederix et al. 2003).

The concept of substrate selectivity is rather new. Its biochemical basis remains as yet unknown. Xylanases within the same GH family and thus with similar specificity differ substantially in substrate selectivity and the classification of GH family, alone, is not sufficient to predict the breakdown of insoluble AXs by xylanases (Moers et al. 2005; Bonnin et al. 2006). Some lines of evidences suggest that the presence of surface exposed aromatic residues can play a role in xylanase substrate selectivity (Moers et al. 2007). Futher factors potentially influencing the degradation of AX populations in situ are reviewed below.

Occurrence of secondary binding sites

Besides the catalytic domain, xylanases (mainly from GH10 families) are frequently linked to one or more noncatalytic substrate binding domain at their *N*- or *C*-terminal ends (Table 1). Their primary function is to increase the enzyme concentration of the substrate thus enhancing the efficiency of the degradation. Xylan binding domains (XBDs), cellulose binding domains (CBDs), and other sugar binding domains are referred to as carbohydrates-binding modules (CBMs) that comprise a diverse group of non-catalytic protein modules that are associated with glycoside-hydrolysing or -modifying enzymes (for a review, see Boraston et al. 2004). Similar to the catalytic modules of GH, CBMs can be further divided into families based on amino acid sequence similarity. There are currently 49 defined families of CBMs and these CBMs displaying variation in ligand specificity. Based on structural and functional similarities these protein modules are grouped into three types: surface binding CBMs (type A), glycan-chain binding CBMs (type B) and small-sugar binding CBMs (type C) (for a review, see Blake et al. 2006).

Linker regions display structural flexibility thereby maximizing substrate accessibility when the enzymes are bound to the plant cell wall via CBMs. In total, the 3D structures of 15 XBD from different CBM families and different types associated with xylanases from GH10, 11 and 43 are now known (http://afmb.cnrs-mrs.fr/CAZY/, Table 1). The β-sandwich fold is predominant among XBDs except for CBM-10 and

CBM-13 that display an OB-fold (oligonucleotide/oligosaccharide binding-fold) and a β-trefoil fold, respectively. Xylan-binding properties have been reported for several type B CBMs in families 2, 4, 6, 15, 22, and 36. They are described as groove or clefts, and comprise several subsites able to accommodate the individual sugar units of the polymeric ligand. Biochemical studies frequently demonstrate increase affinities up to hexasaccharides and negligible interaction with oligosaccharides with a DP of three or less. This type B CBM is equipped to interact with individual glycan chains rather than crystalline surfaces as observed for type A CBM. The nature and number of these CBMs may contribute to xylanase specificity.

In *Cellvibrio japonicus*, five different xylanases (*Cj*Xyn10A, *Cj*Xyn10C, *Cj*Xyn10D, *Cj*Xyn11A and *Cj*Xyn11B) displaying differences in specificities and in the type of CBM act hierarchically. *Cj*Xyn10A is secreted into the culture medium and by binding to the plant cell wall via its crystalline CBM-10 (type A) would be responsible for the degradation of plant cell wall xylans. The further release of soluble xylan and long *xylo*-oligosaccharides would then be hydrolysed by *Cj*Xyn10C into *xylo*-oligosaccharides with a DP > 6, the role of its CBM-15 (type B) being described as a product capture system, thus restricting the diffusion of large *xylo*-oligosaccharides into the environment (Pell et al. 2004). Moreover, addition of type B CBMs to single domain enzymes increased activity of chimeric xylanases towards either soluble (Kittur et al. 2003) or insoluble xylan (Mamo et al. 2007). GH10 xylanase from *Streptomyces olivaceoviridis* contains a family 13 CBM (type C) that binds xylose or *xylo*-oligosaccharides at its three xylan binding sites but its mode of action is effectively used by xylanase to bind long substrate (Fujimoto et al. 2002). The low affinity of the individual binding sites indicates that XBD is able to release bound xylan or *xylo*-oligosaccharides relatively easily. This XBD may bind insoluble xylan by a multi-recognition process involving the triple low affinity binding sites (Fujimoto et al. 2002). Thus, fusions to XBD clearly influence xylanases specificities/efficiencies in situ and may affect their functionality in the degradation of AX.

In single domain xylanases, the presence of a xylan-specific secondary binding site (SBS) can compensate for the lack of CBM to enhance substrate specificity and affinity of the *B. circulans* GH11 xylanase (Ludwiczek et al. 2007). Substrates bound to non-active site surface regions of two single-domain GH8 and GH10 xylanases were also observed by X-ray crystallography, albeit without any demonstrated functional relevance (Schmidt et al. 1998; De Vos et al. 2006). The existence of a non-productive second ligand binding site was recently reported at the surface of two GH11 xylanases from *A. niger* and *B. subtilis* (Vandermarliere et al. 2008). Although such secondary sites may bind small oligosaccharides weakly, in synergy with active site residues they can significantly enhance the specificity and affinity of a xylanase for its natural cell-wall substrates. Furthermore, these carbohydrate binding modules/sites may play a role in dictating substrate selectivity of these enzymes towards WE-AX and WU-AX, as suggested by mutational studies of *B. subtilis* xylanase (Moers et al. 2007).

Sensitivity to proteinaceous inhibitors

Xylanase activity can be affected by the presence of proteinaceous inhibitors in cereals (rye, barley, maize, rice, durum and bread wheat) (Goesaert et al. 2004). To date, the xylanase inhibitors from wheat, XIP (xylanase inhibtor protein)-type (Juge et al. 2004) and the TAXI (*Triticum aestivum* xylanase inhibitor)-type (Gebruers et al. 2004), are the best characterised in terms of structure and function (for a review, see Juge and Delcour 2006).

Both GH10 and GH11 xylanases can be the target of the inhibitor XIP-I (Flatman et al. 2002). The inhibition has been reported for a wide range of fungal xylanases with variation in Ki around 300-fold but none of the bacterial enzymes tested so far were found sensitive to XIP-I (Table 2). The strength of the inhibition is independent of the GH family origin since the two tightest-binding complexes are with the GH11 *Botrytis cinerea* (Ki of 2.1 nM) and GH10 *Aspergillus nidulans* (Ki of 9 nM) xylanases. The inhibition is competitive and structural basis for the inhibition specificity of these enzymes came from the crystallisation of XIP-I in complex with GH10 *A. nidulans* (1TA3.pdb) and GH11 *Penicillium funiculosum* (1TE1.pdb) xylanases. GH10 and GH11 bind to independent and opposite sites on the inhibitor molecule (Payan et al. 2004). The strategy

Table 2 Sensitivity of xylanases to the wheat proteinaceous inhibitors XIP, TAXI and TLXI

Source organism	Accession number	GH family	XIP-I	TAXI-I	TAXI-II	TLXI[a]	References
			Ki (nM) when available				
Fungi							
Penicillium funiculosum XynA	Q8WZJ4	7	106	46	46	n.a.	Furniss et al. (2005)
Aspergillus aculeatus	AAE69552	10	No	No	No	No	Flatman et al. (2002), Gebruers et al. (2001)
Aspergillus nidulans	Q00177	10	9	No	No	n.a.	Flatman et al. (2002), Gebruers et al. (2004)
Aspergillus niger	A62445.1	10	Yes	No	No	No	Gebruers et al. (2004)
Aspergillus oryzae	n.a.	10	17	No	No	No	Flatman et al. (2002)
Penicillium funiculosum XynD	CAG25554.1	10	Yes	No	No	No	Furniss et al. (2005)
Penicillium purpurogenum XynA	AAB35129.1	10	n.a.	No	No	No	Gebruers et al. (2004)
Talaromyces emersonii	AF439747	10	No	No	No	n.a.	Gebruers et al. (2003)
Aspergillus niger	P55329	11	317	20	No	(++)	Flatman et al. (2002), Gebruers et al. (2001), Gebruers (2002)
Botrytis cinerea	n.a.	11	2.1	6.0	n.a.	n.a.	Brutus et al. (2005)
Fusarium graminearum XynA & B	n.a.	11	No	Yes	n.a.	n.a.	Beliën et al. (2005)
Neocallimastix patriciarum	P29127	11	No	n.a.	n.a.	n.a.	Payan et al. (2004)
Penicillium funiculosum XynB	Q8JOK5	11	89.7	2.9	No	n.a.	Brutus et al. (2004)
Penicillium funiculosum XynC	Q9HFH0	11	3.4	17	16	(+)	Furniss et al. (2002)
Penicillium griseofulvum XynA	n.a.	11	No	n.a.	n.a.	n.a.	Berrin et al. (2007)
Penicillium griseofulvum XynB	n.a.	11	No	n.a.	n.a.	n.a.	Unpublished results
Penicillium purpurogenum XynB	Z500050.1	11	n.a.	(+++)	(+)	n.a.	Gebruers et al. (2004)
Trichoderma longibrachiatum XYNI	P36217	11	20	(+++)	(+++)	No	Flatman et al. (2002), Gebruers et al. (2004)
Trichoderma longibrachiatum XYNII	P36218	11	Yes	(+++)	(+)	(+++) 65.1	Flatman et al. (2002) Gebruers et al. (2004)
Trichoderma viride	AJ012718	11	610	(+++)	(+++)	(++)	Flatman et al. (2002), Gebruers et al. (2004)
Bacteria							
Bacillus sp.	Q69230	10	No	n.a.	n.a.	n.a.	Flatman et al. (2002)
Pseudomonas fluorescens	n.a.	10	No	n.a.	n.a.	No	Flatman et al. (2002)
Thermobacillus xylaniticus	O69261	10	n.a.	n.a.	n.a.	No	Gebruers et al. (2004)
Bacillus agaradhaerens	CAB42305.1	11	No	n.a.	n.a.	n.a.	Flatman et al. (2002)
Bacillus subtilis XynA	P18429	11	No	17[b] 2.2[c]	12[b] 2.2[c]	No	Flatman et al. (2002);
Fibrobacter succinogens XynA & B	n.a.	11	No	n.a.	n.a.	n.a.	Flatman et al. (2002)

Table 2 continued

Source organism	Accession number	GH family	XIP-I	TAXI-I	TAXI-II	TLXI[a]	References
			Ki (nM) when available				
Rumen micro-organism M6	n.a.	11	No	No*	n.a.	n.a.	Flatman et al. (2002), Fierens et al. (2004)*
Thermobacillus xylaniticus	CAJ87325.1	11	n.a.	n.a.	n.a.	(+)	Gebruers et al. (2004)
Plant							
Triticum aestivum xylanase	n.a.	10	No	No	No	n.a.	Elliott et al. (2003), Gebruers et al. (2003)

[a] Fierens et al. (2007)

[b] Gebruers (2002)

[c] Sorensen, personal communication

(+++), very strong inhibition; (++), intermediate inhibition; (+), weak inhibition

n.a., not available

for inhibition involves substrate-mimetic contacts and interactions occluding the active site (Tahir et al. 2002; Payan et al. 2004). However not all GH10 and GH11 xylanases are inhibited by XIP-I (see Table 2), and the reasons for the lack of inhibition of these xylanases differ with the GH family origin of the enzyme. For GH11 xylanases, the resistance to XIP-I can, to a large extent, be explained by the structural determinants around the "thumb" region of the enzyme, a key determinant of the xylanase-inhibitor interaction (Payan et al. 2004). GH11 xylanases with amino acid insertions in the tip region of "thumb" are more likely to be uninhibited by XIP-I. This is the case of GH11 xylanases from *Neocallimastix patriciarum* (Payan et al. 2004), *Fusarium graminearum* (Beliën et al. 2005) and *P. griseofulvum* (Berrin et al. 2007) (Table 2). Mutational analysis of these uninhibited enzymes verified the importance of the "thumb" region in conferring the ability to interact with XIP-I (Beliën et al. 2007a, b; André-Leroux et al. unpublished). For GH10 xylanases, the lack of inhibition can, at least partly, be due to differences in the loop regions located around the active site. GH10 with insertions in the β/α-loop segments at the carboxyl side of the β-barrel, which interact with XIP-I, are more likely to be uninhibited by XIP-I (Payan et al. 2004).

GH11 xylanases from both bacterial and fungal origin can be target of the TAXI-type xylanase inhibitors (Gebruers et al. 2004). The inhibition by TAXI is complicated by the presence of two distinct forms, TAXI-I and TAXI-II, with different pI values, and different specificities, i.e. not all xylanases are inhibited by both TAXI-I and TAXI-II. TAXI-I- and TAXI-II-type inhibitors occur in two molecular forms, A and B (Gebruers et al. 2004). In addition, the type of inhibition (competitive/noncompetitive) varies depending on the xylanase (Table 2; Juge and Delcour 2006). For example, competitive inhibition was reported for *P. funiculosum* xylanase C with Ki values of 16 and 17 nM for TAXI-I and TAXI-II, respectively (Furniss et al. 2002) whereas *Bacillus subtilis* xylanase is non-competitively inhibited by both TAXI proteins but with a lower Ki value (2.2 nM) and *A. niger* xylanase is competitively inhibited by TAXI-I only with a Ki of 20 nM (Gebruers 2002) (Table 2). TAXI-type inhibitors do not inhibit GH10 xylanases tested so far. The structure of the TAXI-I-*A. niger* xylanase complex (1T6G.pdb) reveals a direct interaction of the inhibitor with the active site region of the enzyme and further substrate-mimicking contacts with binding subsites filling the whole substrate-docking region (Sansen et al. 2004). The observed difference in xylanase specificity between TAXI-I and TAXI-II can be attributed to the *C*-terminal loop (Sansen et al. 2004; Raedschelders et al. 2005; Bourgois et al. 2007). In addition to the GH10 and GH11 xylanases, a GH7 enzyme XYNA from *P. funiculosum* was reported to be inhibited by XIP-I, TAXI-I and TAXI-II with Ki of 106, 46 and 46 nM, respectively using soluble wheat AX as substrate (Furniss et al. 2005).

Recently, a third type of xylanase inhibitor, thaumatin-like xylanase inhibitor protein (TL-XI), was identified in wheat (Fierens et al. 2007). It is a non-competitive inhibitor of a number of GH11

xylanases, but is inactive towards the GH10 xylanases tested so far. The only Ki value (65 nM) of *Trichoderma longibrachiatum* GH11 xylanase was obtained with xylobiose as substrate due to the interaction between TL-XI and AX.

Xylanase proteinaceous inhibitors present in cereals can interfere with enzymes added as processing aids in cereal technology. For example, the bread-making functionalities of GH11 *A. niger* xylanase depend upon its sensitivity towards TAXI- and XIP-type inhibitors (Gebruers et al. 2005). In the presence of XIP-I, GH11 *A. niger* xylanase is inhibited but the result of the inhibition does not appear to be equally distributed between the effects on WU-AX and those on WE-AX. Interestingly, XIP-I has a tendency to bind WU-AX, but not WE-AX, which changed the balance between enzymatic AX solubilisation and AX depolymerisation (Rouau et al. 2006). A recent study demonstrated that the ability to bind AXs is not restricted to XIP (Sancho et al. 2003) but that TAXI and TL-XI are also able to interact with a similar trend i.e., the affinity increased with a decreasing A/X ratio (Fierens et al. 2008). This trend was most clear with the insoluble AXs. As a result, the interaction of these inhibitors with AXs may also affect the substrate selectivity of xylanases.

Conclusions

This review has mainly focused on factors playing a role in xylanases functionality in the degradation of AXs. Although xylanases have been extensively studied, the classification of GH family is not sufficient to predict the breakdown of AXs by xylanases because the mechanisms underlying their substrate selectivity remain unclear. More work is needed to get insights into the mechanisms underpinning recognition and hydrolysis of AXs as well as interactions with endogenous inhibitors present in cereals. Taking into account specific requirements in cereal processing, structure-function studies will allow the production by molecular engineering of novel enzymes targeted to specific industrial applications.

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